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# Oxidative stress based response of a transcriptional regulator, OhrR, from Burkholderia thailandensis

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OXIDATIVE STRESS BASED RESPONSE OF A TRANSCRIPTIONAL  
REGULATOR, OhrR, FROM *BURKHOLDERIA THAILANDENSIS*.

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirement for degree of  
Master of Science

in

The Department of Biological Sciences

by  
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December 2013

Dedicated to,  
My Parents, Aai and Baba  
And  
My advisor, Dr. Anne Grove.

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## ABSTRACT

Being ubiquitous in nature, bacteria are often faced by environmental stresses. The predominant form of stress is oxidative stress, which prevails during host invasion. As one of the host's protective mechanisms, bacteria are bombarded by organic and inorganic oxidants. Several bacterial antioxidant systems are potent enough to scavenge host-derived reactive oxygen species, helping in bacterial survival. Broadly, they are categorized into those specific for inorganic or organic oxidants. Although a lot has been studied about the former, the latter still remains uncharacterized in several organisms.

*Burkholderia thailandensis* is a soil dwelling bacterium, continually under stress from organic exudates released from plants in addition to other biotic compounds. Besides sharing about 80% genetic homology with its pathogenic homologues *B. pseudomallei* and *B. mallei*, the oxidative environment (rich in organic oxidants) it thrives in makes it an interesting model organism for studying the oxidant sensor-responder proteins it possesses and their mechanism of action.

With an emphasis on such proteins, belonging to the MarR family, OhrR (organic hydroperoxide reductase regulator) protein from *B. thailandensis* was characterized using *in vitro* assays. The gene was cloned from *B. thailandensis* genomic DNA and protein expressed in *E. coli*. The protein was found to exist as a homodimer. It

formed a series of reversible, oligomeric species on being treated with hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide as was observed from SDS-PAGE. The reduced form of the protein was observed to be relatively thermostable ( $T_m$  of 63.5 °C), with an appreciable thermal instability observed in OhrR treated with higher concentration of oxidants (5 mM). DNA binding assays revealed specificity of *B. thailandensis* for the promoter region of *ohr*, although the DNA length to which it bound made a difference in the stability of protein-DNA complexes formed and detected using EMSA.

Since OhrR is predicted to control production of organic hydroperoxide reductase, which is important for detoxifying organic hydroperoxides, the initial characterization and oxidant response of *B. thailandensis* OhrR, determined using *in vitro* assays, thus indicate its probable role as a crucial protein among the *B. thailandensis* protein machinery, aiding in bacterial survival.

# INTRODUCTION

## Oxidative Stress

Oxygen ( $O_2$ ) is essential for most organisms living on planet Earth. In addition to being the most abundant element by mass in the Earth's crust, 21% of the total volume of air we breathe constitutes oxygen. Except for certain anaerobic and aerotolerant single-celled organisms, all animals, plants and bacteria require oxygen for carrying out metabolic activities efficiently.

Originally, oxygen was not a part of the Earth's atmosphere. It was only with the evolution of photosynthetic organisms that oxygen began to be formed and replenished in the atmosphere. For a cell's metabolic activities, most of the oxygen from air is useful as found in its diatomic form. However, being chemically reactive, molecular oxygen can dissipate into several unstable species called 'free radicals' that are extremely harmful [1, 2].

A free radical is any elemental species that contains one or more unpaired electrons. This makes it highly unstable and therefore very reactive. They stabilize themselves by reacting with other substrate molecules, usually biomolecules, thereby modifying these molecules and in some cases even rendering them inactive.

Reactive oxygen species (ROS) are free radical species generated from molecular oxygen. They are important for normal functioning of living systems because they play a crucial role in several cellular processes such as apoptosis, cell-proliferation and differentiation [3], in the immune system as defense against invading pathogens[4], in transcriptional regulation [5] are involved in the electron transport chain and in fact are byproducts of aerobic metabolism [6].

However, on the flipside, they are also one of the major causative factors of neurodegenerative and cardiovascular disorders, skin disorders, diabetes and can even lead to cancer progression [2, 7].

Under normal conditions, cells maintain a steady level of free radicals. They do so by balancing the amount of free radicals generated with the cells own enzymatic and non-enzymatic antioxidant (free radical scavengers) levels. This is the sole reason why structural biomolecules such as proteins, lipids and nucleic acids are still stable even in aerobic environments [6]. The problem arises when this balance is disturbed, and the system is not able to proportionally detoxify the amount of free radicals produced. Biological systems are continually challenged with a burst of free radicals either exogenously (due to exposure to solar radiations, xenobiotics, or elevated oxygen levels) and/or endogenously (in phagocytes and/or host's defense response, unorthodox oxidative metabolic cycles, or depleted antioxidants). Such a condition is called 'oxidative stress' [2, 7].

As a result of their ubiquitous nature, prokaryotic organisms are most prevalently exposed to a variety of oxidative environmental conditions. Other than normal circumstances such as aerobic respiration wherein bacteria are exposed to ROS, another common scenario where bacterial cells are exposed to a burst of oxide radicals is when an invading pathogen encounters the primary line of immune response of the plant or animal host. Elevated levels of these free radicals can damage proteins and lipids in bacterial membranes, in addition to interacting with cellular proteins and nucleic acids, ultimately resulting in cell death [8]. Being non-specific in nature, free-radical production also results in host tissue damage.

The expanse of a bacterial infection therefore depends both on the antioxidant response mechanism of the bacteria to the host-derived ROS, and the extent of host tissue damage [9].

With their rapidly evolving antioxidant systems, bacteria are increasingly becoming resistant to oxidant responses in the process causing host tissue damage, making bacterial oxidative stress responses therefore a leading cause of concern for the scientific world.

#### Multiple Antibiotic Resistance Regulators (MarRs)

Prokaryotes dwell in diverse surroundings varying in pH, osmolarity, temperature, and/or nutrient availability, with each condition capable of being highly stressful for the organism [10]. It is intriguing how the bacteria have been and even now are still evolving to deal with each stress condition, having such a basic cellular and metabolic machinery.

In order to survive and proliferate within the host, among the vast array of proteins bacteria possess to deal with stress, are a family of proteins conferring the bacteria antibiotic resistance. These proteins are transcriptional regulators which sense the oxidative environment, and regulate associated expression of genes involved directly or indirectly as antioxidants (enzymatic or non-enzymatic). Thus, it is the antioxidative response of the bacterium that confers it full virulence [9].

First identified in multidrug resistant strains of *Escherichia coli* [11] such family of proteins that help bacteria sense and respond to stressful environmental challenges are the proteins belonging to multiple antibiotic resistance regulator

(MarR) protein family [12]. With more than 12,000 MarR like proteins annotated as produced in bacterial and archaeal species, they seem to be highly prevalent and therefore biologically important. Till date, MarRs have been shown to function as transcriptional regulators and to control the expression of several genes encoding proteins involved in essential cellular processes such as certain metabolic pathways, enzymatic or non-enzymatic antioxidation, end-product degradation and/or export of toxic substances (byproducts of metabolism, drugs, antibiotics, organic solvents and oxidative agents found in household detergents and disinfecting agents) [13, 14] and in some organisms even virulence [15, 16].

#### Structure of a MarR protein

As shown in the first crystal structure of a MarR protein isolated from *E. coli*, proteins belonging to the MarR family exist as dimers and possess features common to DNA binding proteins. They usually adopt a triangular shaped structure (Figure 1) with each subunit composed of six  $\alpha$ -helices and three  $\beta$ -strands assuming an  $\alpha 1 - \alpha 6, \beta 1 - \beta 3$  topology. The N- terminal region of the protein has been found to be involved in protein-protein interactions while it is the C-terminal region, which is actually involved in DNA binding [17, 18]. The structure of each monomeric unit consists of a DNA-binding domain with a characteristic winged helix-turn-helix (wHTH) motif, and an extensive inter-subunit dimerization interface [19]. The terminal residues of both the N- and C-terminal regions form a hydrophobic dimerization interface stabilized by hydrophobic interactions and hydrogen bonding. Just like in humans, where an

elbow directs the movement of our forearm, the dimerization domain is responsible for flexibility of the DNA binding domains.

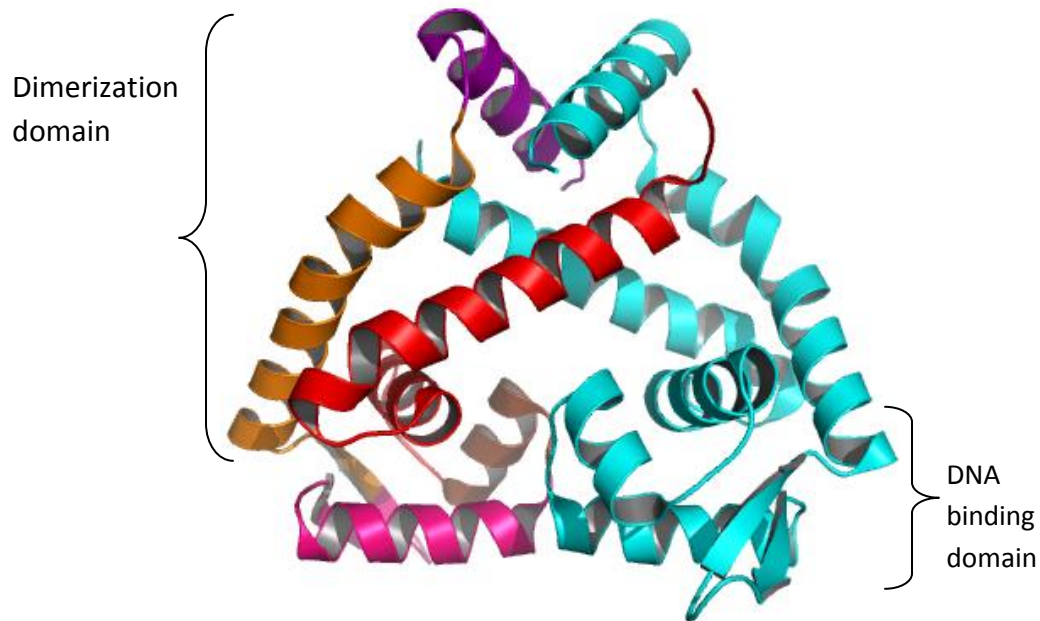


Figure 1. General architecture of a typical MarR homologue with chain A (cyan) and chain B (multicolored) showing different domains. Helices 3 (magenta) and 4 (brown) form the DNA binding domain; helices 1 (red), 5 (orange) and 6 (purple) form the dimerization domain; helix 2 (dark red) lying perpendicular to helix 5 and helix 5 form the connection link between the DNA binding domain and the top part of the MarR structure. Figures 1, 6 and 7 were drawn using Pymol software ([www.pymol.org](http://www.pymol.org)).

The hydrophobic residues in the dimerization domain also help maintain a spatial arrangement between the DNA binding lobes so that each lobe can work independently of the other and ensure better DNA binding [20].

#### DNA binding of MarRs

MarR homologs bind to their cognate DNA sequence as a dimer [11]. The wHTH DNA binding motif (conserved for DNA binding in MarR homologs across all domains of life) has a profound specificity for double-stranded DNA sequences containing inverted repeats, which may or may not be completely palindromic.

Based on previous structural and biochemical studies, the wHTH recognition motif of the DNA binding domain contacts the DNA just as a bird claw clutching onto an object. In almost all MarRs, the recognition helix of the DNA binding domain interlocks with the major groove of DNA first, followed by the secondary association of the winged region with the adjacent minor groove ensuring tight binding [21]. The positively charged residues lining the winged region play a crucial role in DNA binding [22]. Studies also indicate the role of the wing in multimer formation via protein-protein interactions [23].

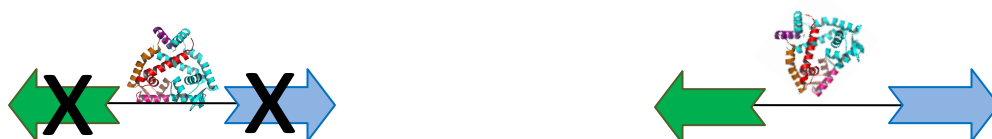


Figure 2. Schematic representation of a MarR gene locus. The green arrow represents the gene coding for a MarR protein while the blue arrow represents the gene that is being regulated. Panel on the left shows the MarR protein repressing gene expression. Panel on the right shows derepression of the gene under regulation

The MarR homologs are encoded by gene loci mostly including two divergent genes, of which one gene encodes for the MarR homolog itself and the other for the gene it is regulating. The intergenic gene sequence separating the two genes contains the MarR-specific cognate DNA. This allows them to regulate both genes, thereby also functioning as auto-regulators [24].

MarRs mainly function as transcriptional repressors [25] although (some/few) of them are also known to behave as transcriptional activators or sometimes both, depending on the dynamics involved in DNA binding [26]. What governs their behavior as gene activators or repressors is the positioning of MarR homologs onto or upstream of the promoter site. Transcriptional repression is achieved by



the MarR protein specifically binding the intergenic DNA sequence, with the sequence usually overlapping promoter region of a gene, thus causing the MarR protein to occupy the transcription start site of the gene (the binding site for RNA polymerase). This can be achieved only when the protein is bound to the DNA in its reduced or unmodified conformation [19, 26] Gene activation or derepression is achieved by the transcriptional factor coming off or remaining loosely bound to the DNA. This helps in making room for the RNA polymerase complex to bind onto the promoter site and continue transcription and therefore gene expression. DNA dissociation is typically a consequence of the MarR protein conformation being changed or modified by an external stimulus, either in the form of binding of a small molecule ligand or oxidation of sensitive cysteine residues [27-30].

#### Organic Hydroperoxide Reductase Regulator (OhrR)

Depending on the environment they are inhabiting, bacteria are always facing harsh oxidative environments, yet are able to survive by virtue of the antioxidative machinery they possess. In addition to several other effectors released in an oxidative stress condition, the major types of ROS generated include superoxide, hydrogen peroxide and organic hydroperoxides. Research till date shows that prokaryotes have an array of transcriptional regulators such as SoxR, OxyR, PerR and OhrR (to name a few), which can directly sense harmful concentrations of ROS and upregulate the expression of genes encoding proteins involved in detoxification [25, 30-33].

In most organisms, the inorganic peroxide species, produced in an oxidative stress condition (superoxides and hydrogen peroxides), are effectively sensed

and detoxified by enzymes encoded and regulated by the SoxRS and OxyR regulons [33]. On the other hand, not much is still clear about proteins responsible for degrading organic hydroperoxides.

Organic hydroperoxides are considered a most toxic form of peroxides, since they not only react with lipid molecules in bacterial membranes and affect membrane fluidity, but their degradation results in generation of more reactive byproducts such as acrolein and malondialdehyde, which can form adducts with proteins and DNA [34]. To prevent their toxic effects, two systems specific for organic hydroperoxide detoxification have been characterized in bacteria, one is alkyl hydroperoxide reductase enzyme (AhpC), a member of the peroxiredoxin family [35], and the other is organic hydroperoxide reductase enzyme (Ohr) [30]. The former is less specific for organic peroxides as it contributes to the reduction of organic hydroperoxides to lesser toxic alcohols in addition to degrading hydrogen peroxides generated endogenously as part of aerobic respiration [36]. Ohr, on the other hand, only participates in the reduction of organic hydroperoxides to alcohols, in a thiol-dependent peroxidase manner and does not respond to any other peroxides. The Ohr protein is regulated by organic hydroperoxide reductase regulator (OhrR), a member of the MarR family, conserved amongst all Gram-negative and Gram-positive bacteria [25, 26, 35]. Initially studied in *Xanthomonas campestris*, Ohr is known to be highly specific for organic hydroperoxides and the *ohr* gene is uniquely upregulated *in vivo* only in the presence of such compounds [25, 30]. The oxidant sensing mechanism of OhrR is facilitated by a family-wide conserved N-terminal active cysteine residue,

which acts as a redox sensor [35, 37, 38] In its reduced form, OhrR protein can bind to its cognate DNA sequence as a clamp (as mentioned before) with the wHTH binding motif facilitating DNA binding. Thus, mostly, in the reduced form, OhrR behaves as a transcriptional repressor [25, 26, 39]. It is only when it is exposed to oxidation, that the OhrR protein changes its confirmation, with the OhrR DNA binding motif undergoing a rotation and dissociating from its cognate DNA (overlapping the *ohr* promoter region) causing consequent *ohr* gene induction [37].

Sequence analysis divides all OhrR proteins into two classes based on the presence/absence of another active cysteine residue. In the first class of proteins with multiple cysteines, the presence of oxidants oxidizes the primary cysteine residue to sulphenic acid, an event that ultimately mediates conformational modification of the protein by intersubunit disulfide bond formation with a neighboring cysteine residue [37]. In proteins with a single cysteine, such as *Bacillus subtilis* (Bs) OhrR and *Streptomyces coelicolor* (Sc) OhrR, the cysteine residue gets oxidized to a sulphenic acid derivative, which does modify the protein and causes weak DNA binding, but is not sufficient to result in *ohr* gene expression [26, 35]. The latter is achieved only when the sulphenic acid intermediate forms a mixed disulphide bond with another intra-subunit thiol group [38]. In some organisms, such as *S. coelicolor*, loosely bound OhrR protein in fact behaves as an activator of *ohrR* [26]. This indicates that although being of the same kind, OhrR protein isolated from different bacteria can exhibit varied DNA

binding properties depending on their amino acid content. So far, only few OhrR proteins have been characterized in detail.

### *Burkholderia thailandensis*

Walter H. Burkholder, was the first to describe one of the species of *Burkholderia* when he first isolated the bacteria from bulb rots caused in rice plants in New York [40]. Although initially placed in the genus *Pseudomonas*, they are now categorized under the genus *Burkholderia* (since 1992) and contain more than 30 different species. Most of the bacteria belonging to this group are opportunistic pathogens, Gram-negative, motile, and obligatory aerobes. They have a large-sized genome with a high degree of plasticity and flexibility that makes survival in varied ecological and stress environments possible, including different kinds of hosts [41]. Additionally, they also have an ability to degrade many chemical compounds [42], which makes some species ecologically and biotechnologically important. However, the degree of pathogenicity associated with others can probably make their ecological benefit debatable.

As a result of their ubiquitous nature, bacteria belonging to the *Burkholderia spp.* have been known to contaminate inanimate objects and therefore are a primary reason for nosocomial infections in hospitals. Their ability to degrade organic and inorganic compounds worsens the case even more, since this property makes disinfectants ineffective against them [43]. Recent studies have shown their prevalence in immunocompromised individuals and those suffering from cystic fibrosis, essentially in patients using various medical devices [44]. Infection with *B. cenocepacia* is particularly common among cystic fibrosis patients.

Of the several species of *Burkholderia* known and studied, of unique interest are the two species – *B. mallei* (Bm) and *B. pseudomallei* (Bp) - listed as category B potential biowarfare agents by the US Centers for Disease Control and Prevention (CDC). They are considered severe health hazards especially to humans due to their aerosol mode of transmission, difficulty in diagnosis and treatment as a result of synergistic infectivity with other disorders, and combined resistance to antibiotics and disinfectants. They have been shown to cause glanders (in horses) and melioidosis (in humans), respectively [45], with a high rate of transfection between the animal and human hosts. Genome comparison between *Burkholderia* spp. shed light on another non-pathogenic species of *Burkholderia* sharing approximately 80% homology with pathogenic Bm and Bp strains, *Burkholderia thailandensis*. It is a non-fermenting, Gram-negative bacilli, essentially non-pathogenic for higher organisms and therefore an ideal model to study proteins responsible for rendering Bm and Bp strains virulent and resistant to antibiotics and disinfectants.

Being a natural inhabitant of soil, *B. thailandensis* is exposed to a variety of oxidants, with a majority of them being organic hydroperoxides present in soil from plant exudates, and antibiotic compounds (fertilizers and pesticides) and other ROS generated as a consequence of oxidative stress. As mentioned earlier, organic hydroperoxides are considered to be the most toxic of all the ROS produced. Although studies have been carried out to understand the role of proteins involved in drug export and peroxide degradation, within the *Burkholderia* species, not much is yet known about organic hydroperoxide

sensitive proteins and how they sense these organic peroxides and trigger their degradation, aiding in bacterial survival.

To better understand this, I decided to study the oxidative stress response of a transcriptional regulator, OhrR, from *B. thailandensis*, predicted to regulate the expression of an organic hydroperoxide reductase (*ohr*) gene, expected to encode an Ohr protein responsible for degrading these organic hydroperoxides.

## MATERIALS AND METHODS

### Cloning and Purification of OhrR

*Burkholderia thailandensis* E264 bacterial strain was purchased from ATCC® (700388D-5™). For extraction of genomic DNA, the bacteria were grown overnight at 37°C in Luria Bertani (LB) broth and DNA was isolated as described (*Current Protocols in Molecular Biology*). The gene encoding *B. thailandensis* OhrR (BTH\_II0598) was amplified from genomic DNA using forward primer 5'-CTTACCGAAAATCTCCCATATGAACGACTCG-3' and reverse primer 5'-CGGACTGGTTCGAACGCCGG-3' (restriction sites underlined). The 453 bp PCR product obtained was then cloned into the NdeI-HindIII restriction sites of pET28b expression vector (Novagen), such that sequence encoding an N-terminal His<sub>6</sub>-Tag preceded the gene. The constructed recombinant plasmid was then transformed into *E. coli* Top10 cells (Invitrogen), confirmed to be correct by DNA sequencing and then re-transformed into *E. coli* BL21 (DE3)pLysS cells for protein expression.

Protein synthesis was initiated by picking up a single colony from a freshly streaked *E. coli* BL21 (DE3)pLysS plate, and growing it overnight at 250 rpm (37°C) in LB broth containing 30 µg/mL kanamycin. For overexpression, the overnight culture was diluted 1:200 times with LB broth containing 30 µg/mL kanamycin and grown for about 2 hours at 250 rpm (37°C) until the O.D.<sub>600</sub> reached about 0.6. Over-expression of protein was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 2 hours. The induced cultures were then cooled down on ice, pelleted and stored at -80°C. For protein extraction, the cells were thawed on ice for about an hour and resuspended in 12 mL ice-cold lysis buffer [300 mM NaCl, 50 mM sodium phosphate

buffer (pH 8.0), 5% glycerol, 300 µg/mL lysozyme, 0.05% Triton-X 100, 2 mM β-mercaptoethanol, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM imidazole]. After incubating the cells in lysis buffer for about 1 hour on ice, the cells were disrupted using a sonicator (3 sets of 7 short pulses over a period of 2 minutes.). This solution was then centrifuged at 9000 rpm for 1 hour. The supernatant was then loaded onto a HIS-Select Nickel Affinity column (Sigma), previously equilibrated with equilibration buffer [300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 5% glycerol]. Further, the column was washed using wash buffer [300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 5% glycerol, 10 mM imidazole, 2 mM β-mercaptoethanol]. The protein was then eluted using elution buffer with gradient concentrations of imidazole in increasing order [300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 5% glycerol, 2 mM β-mercaptoethanol with 15 mM-50 mM-250 mM imidazole]. After confirming the presence of OhrR protein and its purity using SDS-PAGE (12%), specific peak fractions were pooled and dialysed against 1L dialysis buffer [300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 5% glycerol, 2 mM β-mercaptoethanol] for about 12 hours (or overnight). To obtain a higher concentration protein, the dialysed protein was then collected and concentrated using a Millipore concentration column (Centriprep Centrifugal Filter Unit with Ultracel-10 membrane). The concentration of OhrR protein was determined spectrophotometrically at 562 nm, using the BCA protein assay (Pierce). Purity of the protein was confirmed again using SDS-PAGE (12%) followed by staining of the gels using Coomassie Brilliant Blue stain, and protein fractions were stored at -80°C. On an average, depending upon



how long before was the protein stored and purified, prior reduction of OhrR protein may be required before experimentation. 10  $\mu$ M OhrR protein can be reduced with 1-20 mM DTT approximately.

### Gel Filtration

The oligomeric nature of OhrR protein was primarily studied using Gel Filtration. OhrR was easily oxidized in the presence of air. Hence, its oligomeric nature in reduced and oxidized states was determined. For this purpose, a Superose 12 10/300 GL (GE Healthcare) column (10x300 mm) was pre-equilibrated and eluted with gel filtration buffer [150 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 2% glycerol]. As a means of comparison, gel filtration markers (Biorad) were used to create a standard curve. The markers used were bovine serum albumin (66.0 KDa), ovalbumin (44.0 KDa), myoglobin (17.0 KDa) and vitamin B12 (1350 Da). The equation  $K_{av} = (VE-VO)/(VT-VO)$  was used to calculate the  $K_{average}$  ( $K_{av}$ ) of a protein. In this equation, VE, VO and VT represent the retention volume of the protein, void volume of the column and the geometric bed volume of the column respectively.

### Circular Dichroism Spectroscopy

This technique was used to estimate the secondary structure composition of OhrR protein. A Jasco J-815 circular dichroism spectrometer (Jasco Inc.) was used to measure the far-UV circular dichroism spectrum of 10  $\mu$ M OhrR in CD buffer [20 mM NaCl, 12.5 mM sodium phosphate buffer (pH 8.0), 2.5% glycerol, 0.5 mM  $\beta$ -mercaptoethanol] at 20°C. Spectrometric readings were conducted at data pitch points of 1 nm in triplicates using a quartz cuvette with 0.1 cm path length. Secondary structure composition was calculated using the K2D programme from the Dichroweb

DichroWeb [46]. The goodness of fit for the CD spectrum obtained was determined from the NRMSD value of 0.08 with a maximum error of 0.182.

#### Effect of oxidants on OhrR

To understand the effect of organic and inorganic oxidizing agents on OhrR *in vitro*, 5  $\mu$ M protein (reduced) was treated with increasing concentrations of hydrogen peroxide, cumene hydroperoxide and tertiary butyl hydroperoxide for 15 minutes at room temperature. Air-oxidized OhrR and protein sample treated with dithiothretol (DTT) were used as controls. The reactions were terminated by adding sample buffer without any reducing agent (DTT/ $\beta$ -mercaptoethanol) making up the total volume to 10  $\mu$ L. Protein samples were then boiled and subjected to electrophoresis on a 12% SDS-polyacrylamide gel and observed by staining the gels using Coomassie Brilliant blue stain [15].

#### Effect of transition metals on OhrR

To understand the effect of transition metals on *B. thailandensis* OhrR *in vitro*, 10  $\mu$ M protein (reduced) was treated with increasing concentrations of Cu (II), Co (II), or Zn (II) for 15 minutes at room temperature. Air-oxidized OhrR and protein sample treated with dithiothretol (DTT) were used as controls. The reactions were terminated by adding sample buffer without any reducing agent (DTT/ $\beta$ -mercaptoethanol) making up the total volume to 10  $\mu$ L. Protein samples were then boiled and subjected to electrophoresis on a 12% SDS-polyacrylamide gel and observed by staining the gels using Coomassie Brilliant blue stain [47].

### Reversibility assay to study restoration of reduced OhrR

This assay was mainly carried out to assess if the reduced state of OhrR can be restored after oxidation, in order to understand the reversible nature of disulfide bonds formed. For this experiment, 5  $\mu\text{M}$  protein (reduced) was treated with 100  $\mu\text{M}$  of hydrogen peroxide, cumene hydroperoxide and tert-butyl hydroperoxide separately for 15 minutes. Air-oxidized OhrR and protein sample treated with dithiothreitol (DTT) were used as controls. The samples to be restored were reduced with 50 mM DTT and incubated at room temperature for 15 minutes. The reactions were terminated by adding sample buffer without any reducing agent (DTT/ $\beta$ -mercaptoethanol) making up the total volume to 10  $\mu\text{L}$ . Protein samples were then boiled and subjected to electrophoresis on a 12% SDS-polyacrylamide gel and observed by staining the gels using Coomassie Brilliant blue stain [15].

### Glutaraldehyde crosslinking

Protein and glutaraldehyde were combined in buffers free from amines. A 1% glutaraldehyde solution was freshly prepared in 50 mM sodium phosphate buffer (pH 8.0). OhrR protein treated with dithiothreitol (DTT) was used as a control. For glutaraldehyde treatment, 10  $\mu\text{M}$  protein was treated with increasing concentrations of glutaraldehyde and incubated for 20 minutes at room temperature. The reaction was terminated by adding sample buffer without any reducing agent (DTT/ $\beta$ -mercaptoethanol). Protein samples were then subjected to electrophoresis on a 12% SDS-polyacrylamide gel and observed by staining the gels using Coomassie Brilliant blue stain.

## DNA Binding Assays

The operator DNA present in the promoter region of *B. thailandensis ohr* (BTH\_II0597) was amplified from *B. thailandensis* E264 genomic DNA using primers OhrR-F (5'-GTCCTTCATTCTGAAGAATCGCGCCCGCG-3') and OhrR-R (5'-CGGTGGAAATATAGCGTGCCAATAATTAGTGTG-3'). The resulting 73 bp operator DNA (*ohrO-l*) constituted a part of the DNA sequence between adjacent OhrR and *ohr* genes. It was selected based on its similarity with the DNA binding sites of known OhrR proteins. The PCR product was gel purified and solubilized in TE' buffer. Phenol-chloroform extraction can also be carried out after gel extraction for higher yield of the product. Two pmoles of the PCR product was used for 5'-end labeling with <sup>32</sup>P-ATP and T4-polynucleotide kinase for EMSA. In order to narrow down the specific DNA binding site, a shorter DNA sequence 32 bp in length (*ohrO-s*) was commercially synthesized (Operon) and purified using denaturing gels as described [48]. Phenol-chloroform extraction of the oligonucleotides was then carried out followed by solubilizing the oligos in TE' buffer. The respective forward and reverse oligos were annealed overnight from a temperature range of 90°C to room temperature (with the temperature changing at a slow rate). Two pmoles of ds oligos were then used for 5'-end labeling with <sup>32</sup>P-ATP and T4-polynucleotide kinase for EMSA.

DNA binding of OhrR was studied using electrophoretic mobility shift assays (EMSA). To determine the half maximal saturation of OhrR protein, EMSA using 8% (w/v) polyacrylamide gel (39:1 acrylamide:bisacrylamide) in 0.5X Tris-Borate-EDTA (TBE) buffer was carried out. <sup>32</sup>P-labeled *ohrR-l* DNA (0.1 nM) was incubated with

increasing concentrations of OhrR protein in binding buffer [25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.1 mM disodium EDTA, 5 mM dithiothreitol (DTT), 0.05% Brij58, 50 µg/mL bovine serum albumin (BSA) and 0.8% glycerol], and non-specific DNA (2.0 nM/reaction, linearized pET28b) at room temperature (25°C) for 1 hour. After the gel was pre-run for 30 minutes at 10V cm<sup>-1</sup> in 0.5X TBE buffer at room temperature, the samples were loaded onto the gel and run at 10V cm<sup>-1</sup> for 1 hour. The gel was then dried and exposed to phosphor screens and visualized using a Storm 840 phosphorimager (GE healthcare). ImageQuant 5.1 software was used to analyse the data.

Site-specific DNA binding of OhrR protein was studied using a competition assay in which <sup>32</sup>P labeled *ohrO-l* DNA was competed against increasing concentrations of unlabeled *ohrO-l* (competitor 1) and *ohrO-s* (competitor 2). OhrR protein concentration was maintained at 2.0 nM based on the half maximal saturation value of the protein. Non-specific DNA (1.125 nM, linearized pET28b) was added in each reaction. The rest of the EMSA conditions and gel development protocol were maintained same as before.

EMSA to determine DNA binding affinity of OhrR to *ohrO-l* and *ohrO-s* (maintaining common EMSA conditions) was carried out by titrating increasing concentrations of OhrR protein with <sup>32</sup>P-labeled *ohrO-l* and *ohrO-s* DNA (0.05 nM). The same range of protein concentrations were used for titrating both DNA for better comparison. Non-specific DNA (0.84 nM, linearized pUC18) was added in each reaction. The rest of the EMSA conditions and gel development protocol was followed same as before.

### Thermal Stability Assay

The thermal stability of OhrR protein, in various oxidative environments, was measured over a temperature range of 5°C – 94°C. For this purpose, a 96 well reaction plate was used. All reactions were assembled on ice. Depending on the number of conditions to be tested, 6 µM OhrR protein was added to a 1X thermal stability assay (TSA) buffer [20 µM Tris (pH 8.0), 20 mM NaCl] mixed with a reference fluorescent dye 5X SYPRO orange (Invitrogen) and distributed in the wells. Autoclaved distilled water was used to make up the volume to 50 µL [49].

The OhrR protein was treated with increasing concentrations of hydrogen peroxide, cumene hydroperoxide and tertiary butyl hydroperoxide. An additional reaction contained OhrR protein treated with 100 µM Cu (II) solution. Air oxidized protein sample of OhrR and reduced fraction of OhrR protein were also included as controls. Respective blanks for each treatment included the TSA buffer (1X), SYPRO Orange dye (5X), specific treatment conditions (oxidants/reductant) at their respective concentrations and distilled water.

After combining all reagents, the plate was immediately placed for fluorescence emission measurement over a temperature range of 5°C – 94°C in 1°C increments for 10 seconds using an Applied Biosystems 7500 Real-Time PCR system. SYBR green filter was used for detection. Total fluorescence yield obtained from each sample was corrected by subtracting the measured fluorescence obtained from the respective blanks. The sigmoidal part of the melting curve was fit to a four-parameter sigmoidal equation using Sigma Plot 9. The experiment was performed in triplicates and at three independent times for accurate measurements.

## RESULTS AND DISCUSSIONS

### Characterization of OhrR

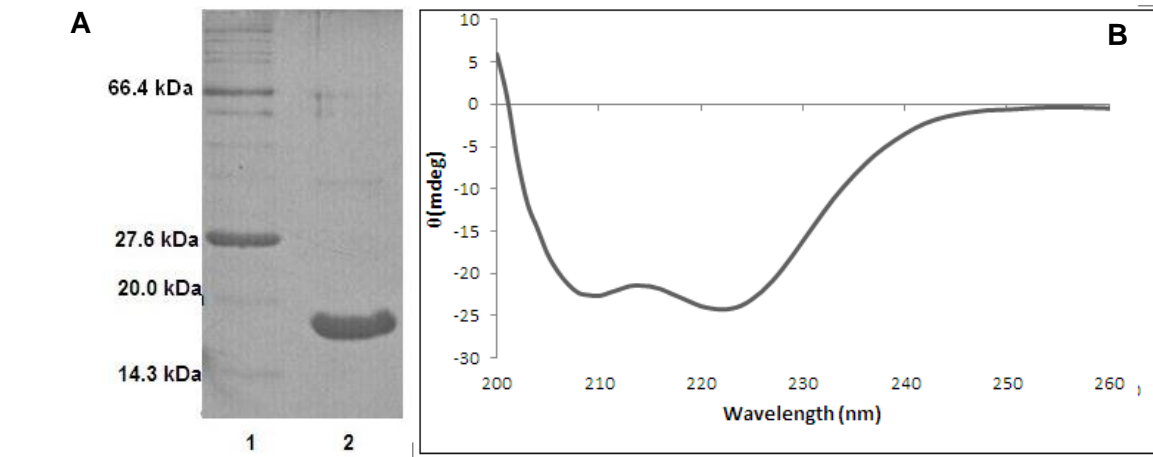


Figure 3A. Purified OhrR resolved as a monomer in 12% SDS-PAGE gel. Lane 1, molecular weight marker (NEB; Mw indicated on the left); Lane 2, Purified OhrR. B. Far-UV CD spectra of purified OhrR (reduced state).

The OhrR gene was amplified from the genomic DNA of *Burkholderia thailandensis* and cloned into an expression plasmid pET28b and expressed in *E. coli* BL21(DE3) cells [26, 39]. Being a small protein and easily soluble, OhrR was well-expressed in *E. coli* BL 21(DE3) cells and purified to apparent homogeneity using a Ni-affinity column. SDS-PAGE was carried out to determine the purity and molecular weight of OhrR protein. The protein was found to be more than 90% pure and resolved on the gel close to 20.0 kDa (Figure 3A), which was consistent with the calculated monomeric weight of recombinant OhrR protein (19.17 kDa). Far-UV circular dichroism spectra showed that the secondary structure composition of OhrR was about 54%  $\alpha$ -helices, 12%  $\beta$ -sheets and 34% random coils (Figure 3B), based on the secondary structure composition estimated by K2D algorithm (Dichroweb) [46]. This was found to be similar to a typical MarR protein containing 58%  $\alpha$ -helices, 12%  $\beta$ -sheets and 34% random coils [27].

Size exclusion chromatography separates protein molecules based on their oligomeric size and shape, in their native state (as purified). Crystallographic studies of MarR proteins [31, 50] reveal that proteins belonging to this family mainly exist as dimers. OhrR proteins function as oxidant sensors, and they change their conformation on being exposed to oxidants [39, 50]. The conserved cysteine residues in these proteins are responsible for sensing the oxidative environment and changing the protein conformation into numerous higher oligomeric states. In order to determine if the same holds true for OhrR protein obtained from *B. thailandensis*, both the reduced and oxidized samples of purified OhrR protein were run on a size-exclusion column. It was observed that the reduced and oxidized fractions of OhrR protein, both eluted at approximately 34.0 kDa (Figure 4B) from the gel filtration column, which was close to the expected molecular weight of the dimeric recombinant OhrR protein (38.34 kDa, as calculated from its DNA sequence).

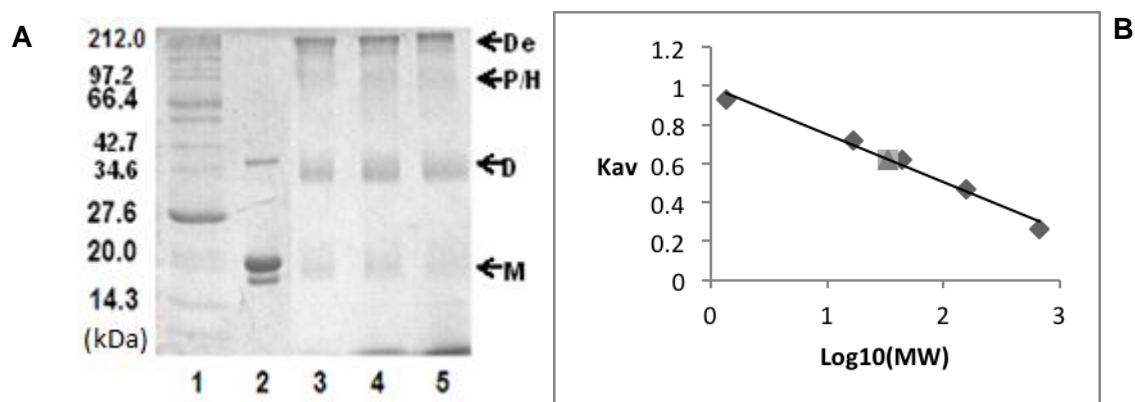


Figure 4. Glutaraldehyde crosslinking of OhrR monomeric units, where 'M' represents monomeric OhrR species, 'D' dimeric, 'P' pentameric, 'H' hexameric and 'De' decameric. Lane 1, molecular weight marker (NEB); Lanes 2-5, 3.8  $\mu$ g OhrR protein; Lane 2, OhrR (reduced); Lanes 3-5, OhrR treated with 0.2%, 0.4% and 0.6% glutaraldehyde, respectively. B. Gel filtration analysis of OhrR. The standard curve was generated by plotting the  $K_{average}$  of molecular weight standards (diamonds) as a function of  $\text{Log}_{10}(\text{MW})$ . The  $K_{average}$  of reduced and oxidized OhrR are shown as a faint grey square and grey triangle respectively.



The fact that reduced OhrR protein also eluted close to its dimeric molecular weight led us to the conclusion that conserved cysteine residues in OhrR protein from *B. thailandensis* might be responsible for sensing oxidants and introducing a conformational change in the protein, but are not involved in oligomerising the OhrR monomer units into dimers, indicating non-covalent bonding between monomer units. Based on the desired function, protein-protein interactions could be long-term, stable (eg: requiring association prior to performing a function), or temporary (eg: interacting with each other only momentarily as a catalyst). Some proteins also have a tendency to simply aggregate or form oligomers under various oxidative conditions. Chemical crosslinking can be used to understand protein-protein interactions *in vitro*. The technique involves formation of a covalent bond between two residues within a protein, yielding dimeric species if intermolecular bonds from each unit are introduced on reaction with an artificial chemical crosslinker [51].

In order to confirm the gel filtration results in understanding the oligomerization nature of OhrR, chemical crosslinking of OhrR monomer units was carried out using glutaraldehyde as the chemical cross-linker; this reagent crosslinks lysine residues. SDS-PAGE results revealed the complete conversion of untreated OhrR sample from its monomeric state to a mixed population of very few monomers (M) and predominantly higher oligomeric species of OhrR including dimers (D), unresolved pentamers/hexamers (P/H), and decamers (De). Treatment with 0.2% glutaraldehyde for 15 minutes was sufficient to convert the monomeric OhrR protein into multimers. The untreated OhrR protein (reduced) fraction showed the presence of some dimeric species, as a consequence of air oxidation. This experiment thus was observed to be

consistent with gel filtration results, showing OhrR from *B. thailandensis* to exist as a dimer or associate as a multimer of dimers under specific conditions.

#### Effects of various oxidative environments on OhrR from *B.thailandensis*

Based on literature, 'Ohr-OhrR' system is the second most popular system studied for organic hydroperoxide scavenging in Gram positive and Gram negative bacteria [26, 30]. By virtue of their ubiquitous nature, bacteria are exposed to a variety of oxidants in the event of oxidative stress. Being a soil bacterium, *B. thailandensis*, is constantly bombarded with several organic and inorganic oxidants. Organic hydroperoxides, known to be more toxic of the lot, have yet not been well characterised in *Burkholderia spp.* hence cumene hydroperoxide and tert-butyl hydroperoxides were chosen. A recent study published by Peeters *et al* [52] showed a 30-40 fold upregulation of the *ohr* gene expression along with a 2.5 fold increase in the *OhrR* gene expression in response to hydrogen peroxide in *B. cenocepacia* cells. This was a new phenomenon to be observed for a protein thought to be specific for organic hydroperoxide sensing. Being a part of the same genus, we chose to include hydrogen peroxide as one of the organic oxidants to be studied to probably have an effect on OhrR from *B. thailandensis*.

The effect of oxidants on OhrR protein was studied by treating the reduced fraction of OhrR protein (0.95 µg) with various organic and inorganic oxidants (10 µM – 5 mM) and resolving them in an SDS-PAGE gel. Though not completely converted to multimeric species, an obvious shift in the oligomeric nature of the protein was observed from its monomeric form (reduced) to its multimeric states with increasing oxidant concentration.

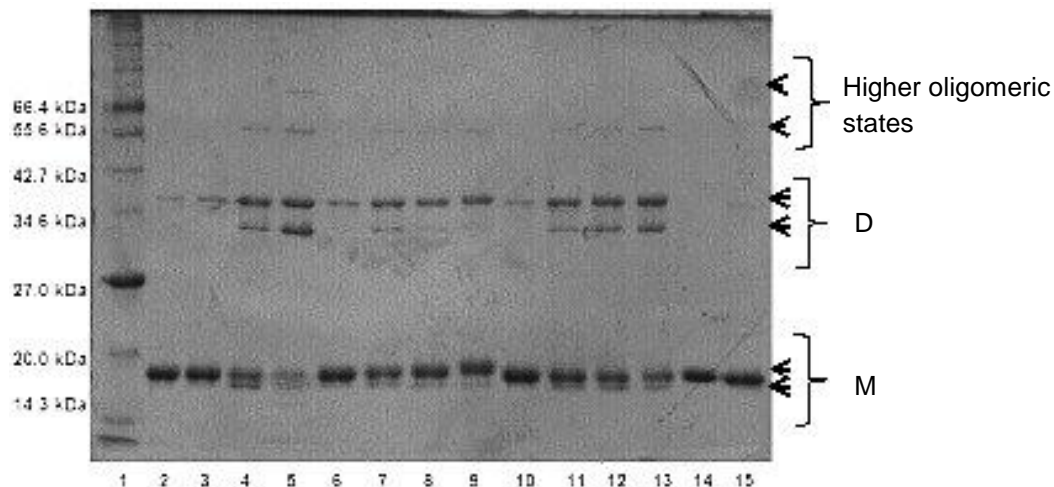


Figure 5. Oxidised and reduced samples of OhrR resolved in non-reducing conditions on 12% SDS-PAGE gel, where 'M' represents monomeric OhrR species and 'D' dimeric. Lane 1, molecular weight marker (NEB); Lanes 2-15 contain 0.95 µg OhrR protein (initially reduced with DTT).

Lanes 2-5 represent OhrR protein treated with 10 µM, 100 µM, 1 mM and 5 mM respectively of hydrogen peroxide;

Lanes 6-9 represent OhrR protein treated with 10 µM, 100 µM, 1 mM and 5 mM respectively of cumene hydroperoxide;

Lanes 10-13 represent OhrR protein treated with 10 µM, 100 µM, 1 mM and 5 mM respectively of tert-butyl hydroperoxide;

Lane 14 – OhrR protein (reduced)

Lane 15 – air oxidized OhrR protein

Treatment with all the three oxidants resulted in formation of dimeric species at 10 µM oxidant concentrations. Hydrogen peroxide caused formation of OhrR multimers at 1 mM and higher concentration, while cumene hydroperoxide and tert-butyl hydroperoxide caused multimers to form at 100 µM concentration.

In comparison to the organic hydroperoxides, hydrogen peroxide caused relatively more multimer formation with a significant disappearance of the monomer band at the highest oxidant concentration (5 mM) probably indicating a global oxidative damage to the protein, caused as a result of small-sized oxidative radicals produced

by hydrogen peroxide. On the other hand, cumene hydroperoxide and tert-butyl hydroperoxide showed a higher population of dimeric species as compared to multimers. This could possibly be as a result of local, site-specific oxidation of cysteines by large-sized organic oxidant molecules in comparison to the inorganic molecules. The probable model of OhrR (Figure 6) shows two cysteines (one from each monomeric chain) symmetrically disposed on either side of the central axis.

In the case of hydrogen peroxide and tert-butyl hydroperoxide, a mixed population of OhrR oxidation products were observed ranging from monomers and dimers to multimers. The dimer band was observed appearing as a doublet, probably because of the type either one of the following possible inter-subunit disulfide formations a) One or both of the Cys121 involved in inter-molecular bonding with the respective Cys16 of the opposing subunit or, b) Cys16 of both the subunits (lying near the central axis of the structure) involved in inter-molecular bonding. Dimers associated by a single disulfide linkage might migrate slower on the gel as compared to those associated with more than one disulfide bond, which would separate faster on the gel by virtue of a more rigid structure. Hence, the doublet.

On the other hand, at higher oxidant concentrations, multimeric species might have been formed via multiple inter-molecular disulfide bonding between one or both of the protruding cysteines (Cys121) with one or both of the equivalent cysteines (Cys121) of neighboring protein molecules (chain-like assembly) or a due to a combination of an inter-subunit disulfide bond (either Cys16-cys16 or Cys121-Cys16) within a protein molecule and a simultaneous chain-like intersubunit disulfide bond between

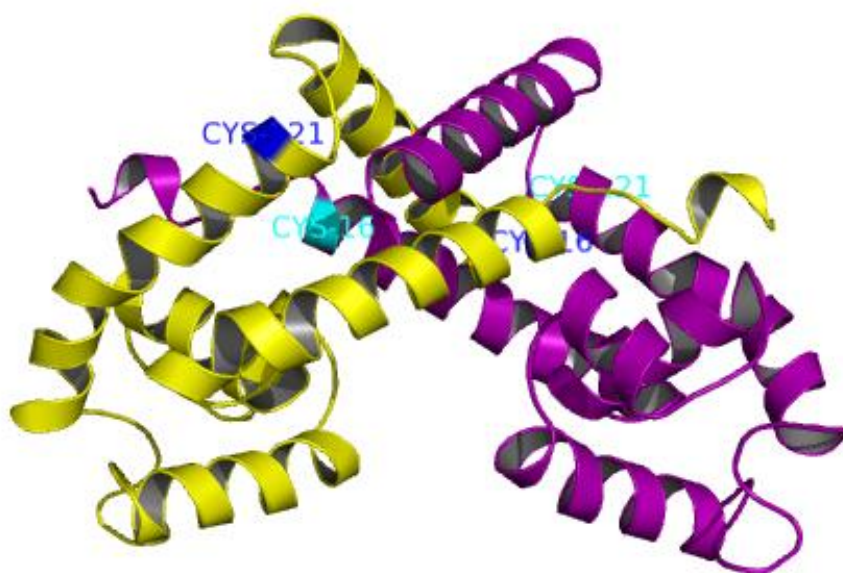


Figure 6. Modeled structure of *B. thailandensis* OhrR (Swiss-model server) with one subunit colored purple with cysteines marked in cyan and the other subunit yellow with cysteines marked in blue.

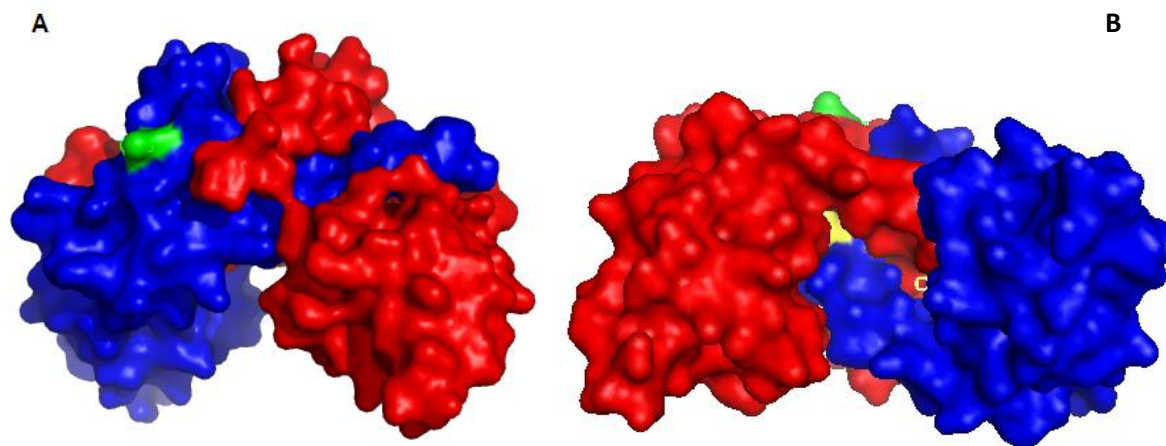


Figure 7A. Surface rendering of the modeled *B. thailandensis* OhrR with Cys121 (green) shown protruding out. B. Dorsal view of the modeled *B. thailandensis* OhrR with Cys121 (green) shown protruding out and Cys16 (yellow) buried in the central axial pocket.

the free Cys121 of the same protein molecule with Cys121 of the neighbor. In contrast, cumene hydroperoxide-treated OhrR samples showed only faint traces of

the fast-moving dimer band, instead showing a gradual increase in the slow moving dimeric OhrR band intensity, indicating an increase in dimer species associated via single disulfide linkage. This could probably be occurring as a result of preferred oxidation of Cys16 by cumene hydroperoxide in the process resulting in asymmetric inter-molecular disulfide linkage between Cys16 and Cys121 on either of the two sides of the OhrR structure (relative to the central axis). Absence of multimers suggests preferred oxidation of buried cysteines by cumene hydroperoxide resulting in only specific dimers. At higher oxidant concentrations, some intermediary, incompletely oxidized conformations of OhrR were also observed migrating faster than the monomeric band of the protein. Although intra-molecular disulfide bonding might be rare as a result of the two cysteines being quite far apart from each other (34.49 Å), the faster moving band near the monomer might be a consequence of such an event. Disulfide linkage confers a rigid structure upon the protein making it move faster on the gel. The untreated OhrR protein (reduced) fraction showed the presence of some dimeric species, as a consequence of air oxidation.

Overall it was observed that oxidation of OhrR with both organic or inorganic oxidants resulted in protein dimerization. For cumene hydroperoxide, dimeric species were the primary oxidation product, on the other hand, hydrogen peroxide and tert-butyl hydroperoxide yielded additional oxidation products. This might probably be due to the specificity of OhrR protein to organic hydroperoxides (OHPs) especially cumene hydroperoxide by virtue of its highly hydrophobic nature and small size in comparison with tert-butyl hydroperoxide. Structural studies on OhrR isolated from *X. campestris*, reveals the presence of an OHP binding pocket lined by hydrophobic residues (valine

methionine and proline) and a small hollow cleft just enough in size for cumene hydroperoxide to fit [50].

#### Metals as oxidants.

As compared to other elements, transition metals occur at a relatively lower concentration in the living system, yet they play a crucial role in various metabolic and signaling pathways. Transition metals can exist in several oxidation states, making them useful catalysts for oxido-reductive. Although very little has yet been studied about metalloregulatory MarR proteins, with the exception of two studies carried out in the past – one of the AdcR protein from *S. pneumonia* and the other published recently showing a MarR regulator responding to copper [47, 53], we thought it would be interesting to study the metal binding nature of OhrR from *B. thailandensis*, if any.

By virtue of their electronic distribution and redox properties, transition metals play a mediatory role in catalyzing free radical reactions. The problem arises when their cellular concentration becomes uncontrolled, and they start catalyzing unwanted free radical reactions. Their reactive property allows their escaping easy from the cell's own homeostasis control [54]. Thus, transition metals are prime players in oxidative stress.

To test the effect of metals on OhrR, 10  $\mu$ M OhrR protein was treated with 1  $\mu$ M - 90  $\mu$ M of Cu (II), CO (II), and Zn (II) and resolved on an SDS-PAGE gel. It was observed

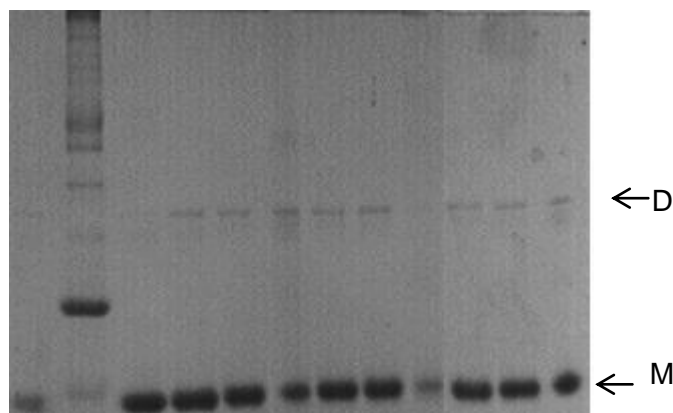


Figure 8. Metal treated samples of OhrR resolved in non-reducing SDS-PAGE gel (12%) stained using Coomassie Brilliant Blue stain, where 'M' represents monomeric OhrR species and 'D' dimeric. Lane 1, Air oxidized OhrR; Lane 2, Molecular weight marker (NEB); Lanes 1, 3-15 contain 1.9  $\mu$ g OhrR protein (initially reduced with DTT). Lane 3, OhrR protein (reduced); Lanes 4-6, 1  $\mu$ M, 10  $\mu$ M and 90  $\mu$ M of Cu (II) respectively; Lanes 7-9, 1  $\mu$ M, 10  $\mu$ M and 90  $\mu$ M of Co (II) respectively, Lanes 10-12, 1  $\mu$ M, 10  $\mu$ M and 90  $\mu$ M of Zn (II) respectively.

that all metals in various concentrations showed dimer formation. OhrR has been known to play a role in oxidative stress [25]. Since transition metals mediate free radical synthesis, and OhrR already is a known oxidant sensor, the probable reason for such a non-specific metal-induced oligomer formation in OhrR is metal-mediated free radical synthesis, affecting OhrR conformation instead of the metal/s directly binding the protein ligand site and causing a conformational change.

#### Reductant induced restoration of oxidized OhrR.

In order to understand the redox state of *B. thailandensis* OhrR when purified, we carried out gel filtration and glutaraldehyde crosslinking, which showed that OhrR exists as a non-covalently bonded dimer in its reduced form.



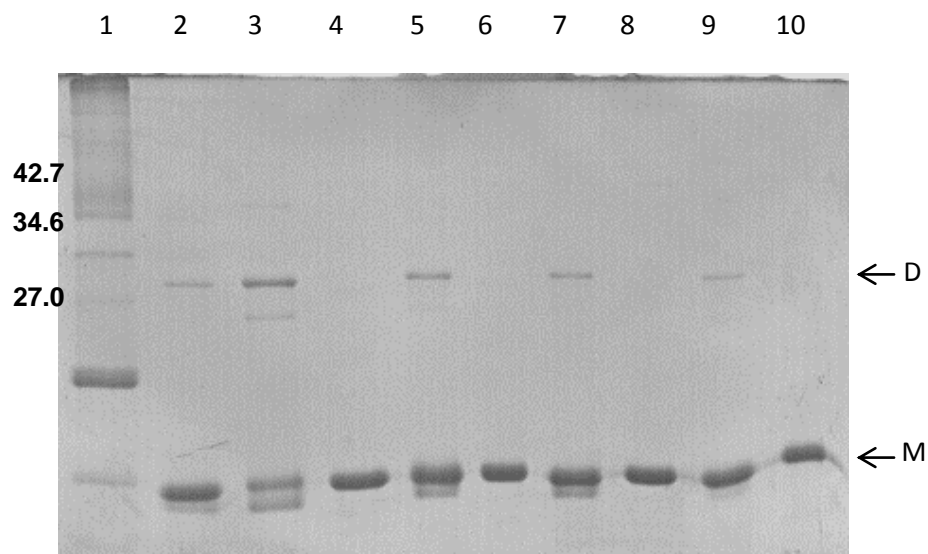


Figure 9. Oxidised and reduced samples of OhrR resolved in non-reducing SDS-PAGE gel (12%) stained using Coomassie Brilliant Blue stain, where 'M' represents monomeric species of OhrR and 'D' dimeric. Lane 1, Molecular weight marker (NEB); Lanes 2-10 contain 0.95 µg OhrR protein (initially reduced with DTT). Lane 2, OhrR protein (reduced); Lane 3, OhrR protein oxidized with 100 µM hydrogen peroxide; Lane 4, OhrR protein initially oxidized with 100 µM hydrogen peroxide, then reduced with 50 mM DTT; Lane 5, OhrR protein oxidized with 100 µM cumene hydroperoxide, Lane 6, OhrR protein initially oxidized with 100 µM cumene hydroperoxide, then reduced with 50 mM DTT; Lane 7, OhrR protein oxidized with 100 µM tert-butyl hydroperoxide; Lane 8, OhrR protein initially oxidized with 100 µM tert-butyl hydroperoxide, then reduced with 50 mM DTT; Lane 9, OhrR protein oxidized with 100 µM Cu (II); Lane 10, OhrR protein initially oxidized with 100 µM Cu (II), then reduced with 50 mM DTT.

Further on, how its redox state changes in various oxidative environments was studied by treating the OhrR protein with various organic and inorganic oxidants and observing formation of dimeric species on SDS-PAGE gels, implying the formation of intermolecular disulfide bonds. To determine if this oxidative condition could be reversed or not, we tried reducing the oxidized OhrR protein samples using DTT.

As observed in Figure 9, the dimer band (observed on treating OhrR with 100 µM concentration of oxidants) disappeared completely with only monomer detectable following reduction (completely reduced state). Reversibility of oxidation was witnessed for all oxidants – hydrogen peroxide, cumene hydroperoxide, and tert-butyl

hydroperoxide. The untreated OhrR protein (reduced) fraction showed the presence of some dimeric species, as a consequence of air oxidation. Since a much higher concentration of DTT was used for reduction, the shift in the oligomeric state of OhrR from dimer to a monomer was rapid. A gradient reduction of oxidized OhrR samples using DTT could be performed in order to study which of the two dimer conformations (relaxed or rigid) is converted to monomeric state first and at what DTT concentrations. This could shine some light on the stability of the various disulfide bonds formed, and probable placement of cysteine residues involved in bonding [15].

#### Thermal Stability of Reduced and Oxidised forms of OhrR

Thermal stability of OhrR protein, in various oxidative conditions, was determined using thermal shift assay. The thermal stability profile was measured using fluorescence spectroscopy. The fluorescent dye used for the purpose, Sypro Orange, is hydrophobic in nature. As the protein unfolds as a function of temperature, the buried hydrophobic residues in the protein get exposed, to which the dye Sypro Orange (whose fluorescence is normally quenched in aqueous solutions by water) binds (via hydrophobic interactions), resulting in an increase in fluorescence.

According to the thermal stability results analysed using Sigma Plot 9 software, purified OhrR (reduced state) was observed to be quite stable with a  $T_m$  of  $63.5 \pm 0.4^\circ\text{C}$  (Figure 10, table 1). This was consistent with other MarR homologues, showing relatively high thermal stability [27, 55]. In comparison to reduced OhrR, air-oxidized OhrR and OhrR treated with low concentrations (100  $\mu\text{M}$ ) of organic and inorganic oxidants, contained only a small percentage of dimer as revealed by the SDS-PAGE gels (Figure 11), but a significant fraction of faster migrating monomer.

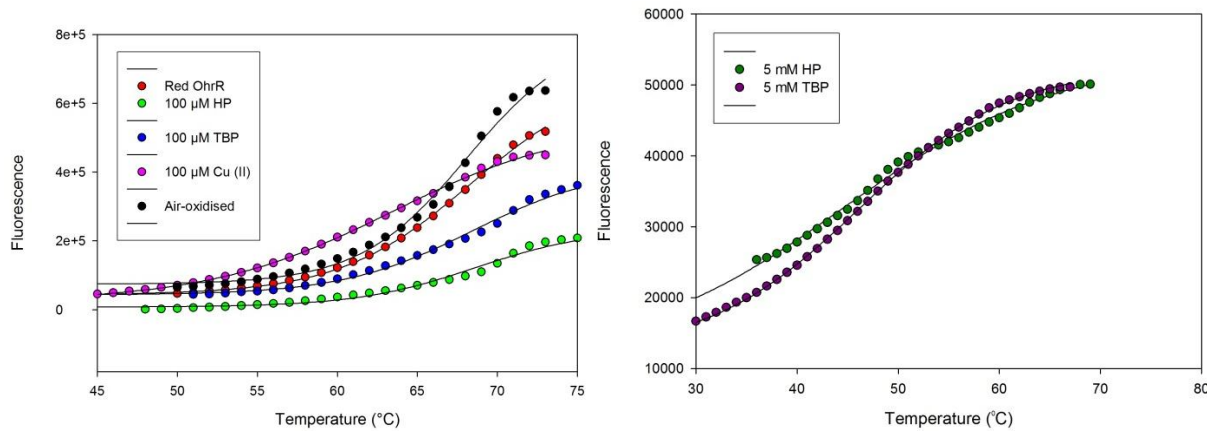


Figure 10. Thermal melts depict fluorescence emission of reduced vs oxidized OhrR, treated with low concentration of oxidants (left panel) and oxidized OhrR, treated with high concentration of oxidants (right panel).

A probable reason for this could be the significant content of non-covalently linked (reduced) OhrR molecules still co-existing with the partly oxidized OhrR protein (also evident in Figure 11), resulting in the measurement of thermal stability of a mixed population of species.

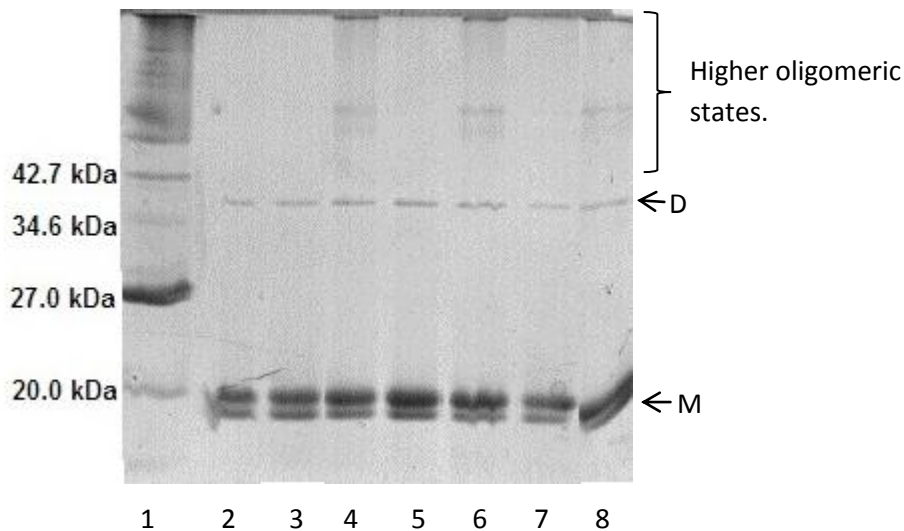


Figure 11. OhrR protein fractions used for assessing thermal stability resolved on SDS-PAGE gel. Oxidant treated samples of OhrR resolved in non-reducing SDS-PAGE gel (12%) stained using Coomassie Brilliant Blue stain, where 'M' represents monomeric species of OhrR and 'D' dimeric. Lane 1, Molecular weight marker (NEB); Lanes 2-8 contain 0.12 μg OhrR protein (initially reduced with DTT); Lanes 3-4, OhrR protein treated with 1 μM hydrogen peroxide, and tert-butyl hydroperoxide respectively; Lanes 5-6, OhrR protein treated with 10 μM hydrogen peroxide, and tert-butyl hydroperoxide respectively; Lanes 7-8,

OhrR protein treated with 100  $\mu$ M hydrogen peroxide and tert-butyl hydroperoxide respectively; Lane 11, Air oxidized OhrR protein.

On the other hand, a remarkable shift in melting temperatures was observed in case of OhrR treated with higher concentrations (5mM and 10 mM).

Table. 1. Comparison of melting temperatures ( $T_m$ ) of oxidized and reduced OhrR.

Conc. Sample	100 $\mu$ M	5mM
Controls	$63.5 \pm 0.4$ (reduced OhrR) $68.5 \pm 0.4$ (air oxidized OhrR)	
HP	$68.8 \pm 0.0$	$43.1 \pm 0.8$
TBP	$69.1 \pm 1.0$	$43.0 \pm 1.4$
Cu(II)	$62.3 \pm 1.5$	-

of organic and inorganic oxidants. Although disulfide bonds are known to increase the stability of a protein [56], the process of disulfide bond formation or rather the expense at which the covalent bond is made could make a difference. Although non-covalently associated OhrR might be expected to be less stable in comparison to a covalently associated OhrR (based on their bond dissociation energies), at high oxidant concentrations, a covalent disulfide bond between monomers could be possibly made at the expense of disrupting a number of bonds otherwise responsible for holding the structure together. On the other hand, although the same might be occurring in a non-covalently held dimer, the

event might be accompanied by other associative bonds holding the structure together adding overall stability to the protein.

Cu (II) treated OhrR also showed a decrease in thermal stability, probably for the same reason as high concentration of oxidants. Transition metals generate free radicals as byproducts predominantly forming hydroxyl radicals, which on account of their small size, reactivity and non-specific mode of action could be responsible in forming non-specific disulfide linkages just like hydrogen peroxide, ultimately resulting in decreased thermal stability.

#### DNA binding properties of *B. thailandensis* OhrR

Previous studies suggest most of the proteins belonging to the MarR family have high specificity for palindromic sequences lying in the intergenic region between divergent genes coding for the MarR protein and the gene whose expression the former regulates [Inoka 15, 27]. Although the DNA binding sites for OhrR proteins vary in different bacteria, a conserved core DNA binding region has been identified in the *ohr* promoter region and found to be rich in inverted 'AATT' repeats [25, 57, 58].

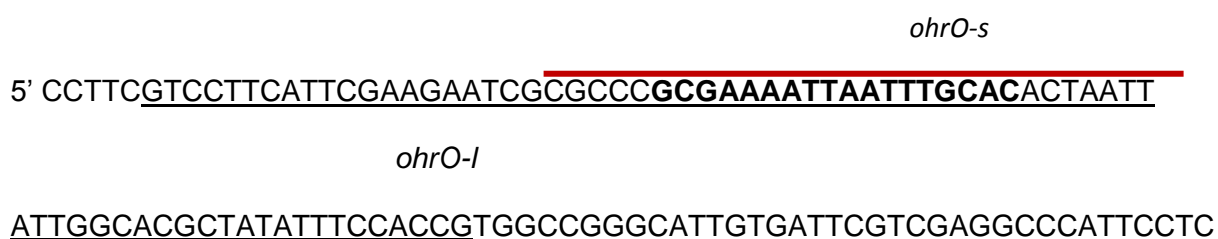


Figure 12. Promoter region of *ohr*. Conserved OhrR DNA binding site in bold. 73 bp *OhrO-l* DNA sequence (underlined), 32 bp *ohrO-s* DNA sequence (selected in red; the oligo was synthesized with the last base changed from T to G to maintain experimental stability).

Based on this knowledge, DNA interaction studies of *B. thailandensis* OhrR were carried out using purified OhrR (reduced state) and a 73 bp long operator DNA

(containing the hypothesized OhrR binding site) named *ohrO-I*, lying in the *ohr* promoter region of the *B. thailandensis* genome.

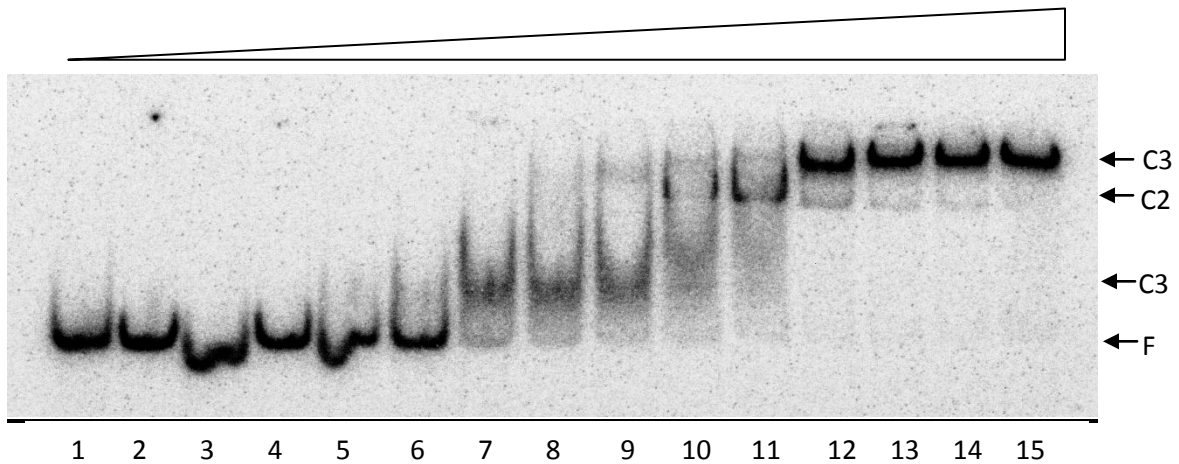


Figure 13. EMSA showing binding of OhrR protein to *ohrO*-long DNA (73 bp). Lanes 2-15 represent labeled 73 bp DNA titrated with increasing concentrations of reduced OhrR protein (10 pM – 2.0  $\mu$ M). 1.6 nM linearized non-specific plasmid DNA (pUC18) was added per reaction. Reaction in Lane 1 contains free DNA. Three detectable complexes (C1, C2, C3) and free DNA (F) are marked as arrows.

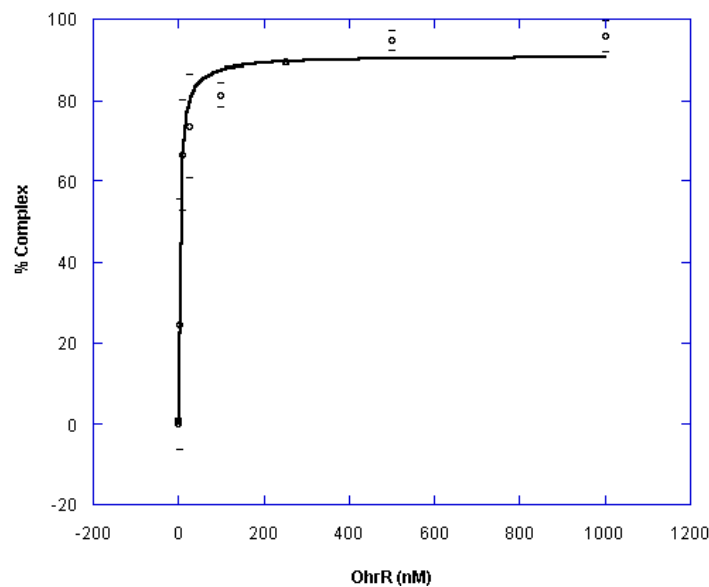


Figure 14. Plot indicating half maximal saturation of 73 bp long DNA sequence to OhrR.

OhrR specifically bound to labeled *ohrO-I* forming three clearly visible complexes as seen in Figure 13. The binding was of high affinity as evidenced by the first OhrR-*ohr-I* complex (C1) appearing at about 1 nM protein concentration (Figure13) in

addition to its apparent half-maximal saturation value of 3.6 nM (Figure 14). Excess of non-specific DNA was added to each reaction in order to ensure stringent binding.

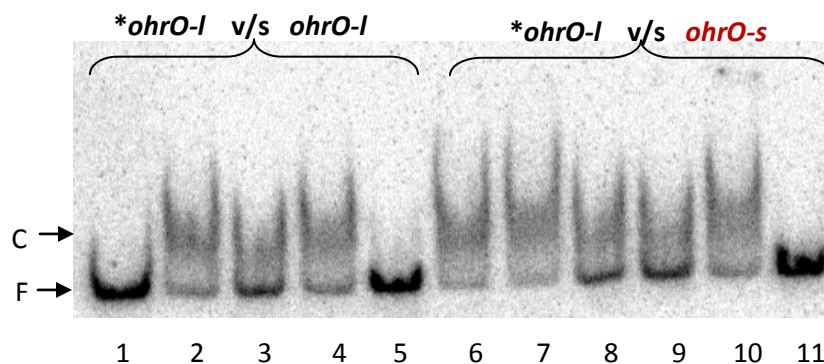


Figure 15. Competitive EMSA showing binding of 2.0 nM OhrR protein to labeled 73 bp DNA segment (within *ohrR-ohr* intergenic region) competed against two competitors; Lanes 3-5: contain increasing concentrations (0.05 nM, 0.1 nM, 1.0 nM) of same unlabeled 73 bp long DNA respectively; Lanes 6-8, 9-11: contain increasing concentrations (0.05 nM, 0.1 nM, 1.0 nM) of unlabeled 32 bp short DNA respectively. Lanes 1-5 and 9-11 contain 1.125 nM linearized non-specific DNA (pET28b).

Specificity of *ohrO-I* sequence to OhrR was confirmed on competing labeled *ohrO-I* against unlabeled *ohrO-I* for binding OhrR. Labeled, unbound *ohrO-I* was retrieved on addition of high concentrations of unlabeled *ohrO-I* (Figure 15). Previous experimental evidence suggests the presence of a single conserved binding site in the *ohr* promoter region. However, formation of C2 and C3 observed on increasing OhrR concentrations (Figure 14) indicated the possibility of another binding site probably existing in the *ohrO-I* DNA sequence.

Once specificity of *B. thailandensis* OhrR for *ohrO-I* (spanning the major part of the *OhrR-ohr* region) was confirmed, narrowing down the binding site/s specifically was attempted. A short 32 bp sequence (*ohrO-s*) containing the hypothesized OhrR binding site flanked by 7 bp on either side was synthetically designed and purified using Urea-Acrylamide gel electrophoresis and extraction method.

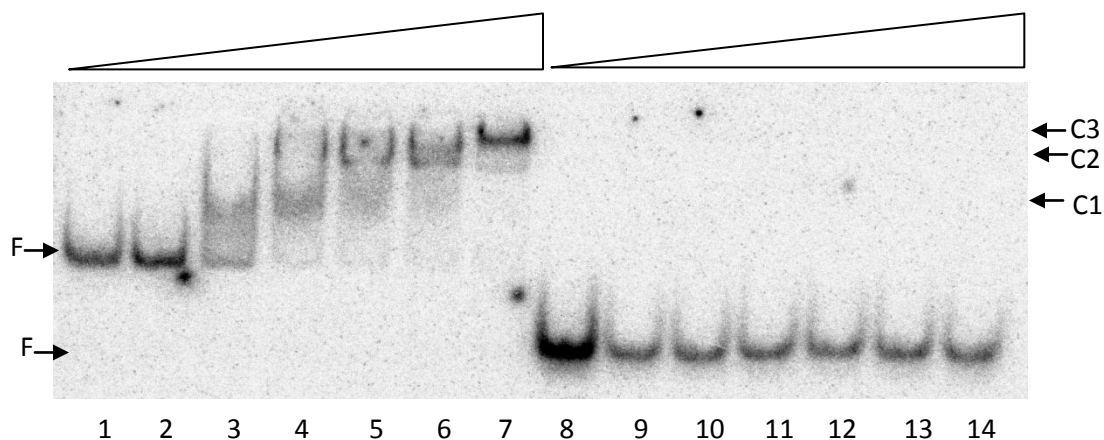


Figure 16. Comparative EMSA showing a contrast in DNA binding of OhrR to the 73 bp labeled long DNA (Lanes 1-7, 0.05 nM) and 32 bp labeled short DNA (Lanes 8-14, 0.05 nM). Titrations of OhrR protein in increasing protein concentrations (0.1 nM – 1.0  $\mu$ M) against the specific labeled DNA have been shown. Lanes 1 and 8 contain free DNA. Lanes 1-14 contain 0.84 nM non-specific linearized plasmid DNA per reaction. Three detectable complexes (C1, C2, C3) are marked as arrows.

To confirm the specificity of *B. thailandensis* OhrR to *ohrO-s*, labeled *ohrO-I* was competed against *ohrO-s* for binding OhrR. Unlabeled *ohrO-s* competed very well with labeled *ohrO-I* with almost complete retrieval of the latter at higher concentrations of the former (Figure 15). The highest concentration of unlabeled *ohrO-s* (1.0 nM) in fact competed against labeled *ohrO-I* to the same degree as unlabeled *ohrO-I* (1.0 nM), suggesting that the conserved 18 bp DNA sequence (hypothesized DNA binding site) is the primary binding site for OhrR.

Although the specificity of OhrR for *ohrO-s* was indicated by competition assay, it was surprising to observe no protein-DNA complex formed on titrating labeled *ohrO-s* with increasing concentrations of OhrR protein (Figure 16), probably a consequence of unstable complexes formed non-detectable by EMSA.

From the EMSA results obtained, the probable reason for unstable complex formation between OhrR and *ohrO-s* could be the DNA length. Although highly specific (with only one conserved DNA binding site being present and not much extra



DNA), *ohrO-s* is probably too short to hold two protein molecules in a stable state to be detected in an EMSA (Figure 16). When the same binding site is lengthened with extra base pairs on either side to generate the 73 bp DNA (*ohrO-l*) it shows better binding to OhrR demonstrated as three detectable protein-DNA complexes (Figures 14 and 16). An argument could be raised with the smeary complex band being non-specific, however, with an excess of linearized non-specific plasmid DNA being present in the reaction, that seems unlikely. The *ohrO-s* site, being highly palindromic and similar to other OhrR binding sites, is probably a more preferred binding site for OhrR. What might be happening is that when the protein concentration is low, OhrR binds the *ohrO-s* site due to preferred binding, but the short length of DNA might only be able to accommodate one protein molecule, appearing as unstable smeary C1 bands (Figure 17).

As the protein concentration increases and more protein molecules are available to stabilize primarily weakly bonded protein, C1 bands are seen to be disappearing and transitioning into a relatively stable tight complex band C2. The gradual transition of



Figure 17. Proposed mechanism of OhrR binding DNA.

smeary faint bands of C1 into C3 via C2 as protein concentration increases (Figure 14) supports this interpretation. This could also mean that the binding of OhrR dimer onto DNA is not ribosome like, but like inverted cups attached end to end in chains, where one protein molecule uses the already bound protein molecule as the anchor.

At very high concentrations, a third complex appears indicating another lower affinity binding site occurring in the promoter region of *ohr OhrR-ohr* intergenic region, which is not surprising as sequence analysis of the promoter reveals another 'AT' rich site with a characteristic 'AATT' repeat, which might be the probable second binding site. These interpretations need to be confirmed by footprinting.

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## **VITA**

Anuja Rajiv Pande, was born in Mumbai, India. Having completed her primary education in Mumbai and partly in Dubai, she completed her undergraduate studies in Life Sciences and Biochemistry, from the University of Mumbai, in the year 2007. During her bachelors tenure, in addition to academic studies, she also had the opportunity of being a part of individual research projects at the collegiate and national levels.

In 2011, she completed her Masters in Biological Sciences, from SVKM's NMIMS University, School of Science and then moved to United States to pursue her second Masters degree at the Department of Biological Sciences at Louisiana State University, Baton Rouge.

In the Spring of 2012, she joined Dr. Anne Grove's lab to study the MarR family of transcriptional regulators responsive to organic hydroperoxides, as part of her Masters programme. Upon completion of her degree she wishes to pursue her doctorate under the guidance of Dr. Anne Grove and carry the research element further.